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ORIGINAL PAPER

Overexpression of the r*FCA* RNA Recognition Motif Affects Morphologies Modifications in Rice (*Oryza sativa* L.)

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Abstract RNA recognition motifs as important regulators of gene expression are highly conserved in animals and plants. The *FCA* floral promotion gene in *Arabidopsis* encodes a protein, containing two RNA recognition motifs (RRM) and a WW protein interaction domain. Here we isolated *FCA* cDNA from rice. *FCA* in rice (r*FCA*) was homologous to *FCA*-gamma of *Arabidopsis* and contained conserved domains. To investigate the function of RRM domain, fragment RRM1 and RRM2 of r*FCA* were introduced into rice subspecies *Oryza sativa* L. *subsp. Indica* var. 9311 and another rice subspecies *Oryza sativa* L. *subsp. Japonica* var. zhonghua11 transformation. Two transgenic lines exhibited similar phenotypes, flowering time delay, seed size and cell volume of transgenic plants was increased. These results showed that constitutive expression of RRMs could regulate cellular size. The patterns of overexpression of two RRM domains and their similar morphologies indicate they may play a same role.

Keywords *FCA* · RRM · Rice (*Oryza sativa* L.) · Cell size

Introduction

In eukaryotic cells, regulation of gene expression at the post-transcriptional level is mainly achieved by proteins, which containing well defined sequence motifs involved in RNA binding. Most RNA-binding proteins contain one or several conserved domains, such as the RNA recognition motif (RRM), the K-homology (KH) motif, RGG (Arg-Gly-Gly) boxes, and double-stranded RNA-binding domains (dsRBDs) (Burd and Dreyfuss 1994). The most widely spread motifs are the RRM. The *Arabidopsis* genome encodes 196 RRM-containing proteins and 26 KH domain proteins (Lorković and Barta

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2002). Regulation is mainly achieved either directly by RNA-binding proteins or indirectly, whereby RNA-binding proteins modulate the function of other regulatory factors. The large variety of possible RNA targets implies the existence of a large number of RNA-binding proteins with different binding specificities. The most abundant nuclear RNA-binding proteins in human cells are collectively termed heterogeneous nuclear ribonucleoproteins (hnRNPs), according to their association with nascent RNA polymerase II transcripts. Molecular cloning of genes encoding hnRNPs led to the discovery of several motifs involved in RNA binding (Burd and Dreyfuss 1994; Swanson 1995). Most RNA binding proteins serve multiple functions within the cell, affecting mRNA splicing, polyadenylation, transport, localization, stability, and/or translation (Ladd and Cooper 2004). The RRM, also known as RNA-binding domain (RBD) or ribonucleoprotein domain (RNP) is one of the most abundant protein domains in eukaryotes and regulate post-transcriptional gene expression (Maris et al. 2005). Expression of higher plant mitochondrial (mt) genes is regulated at the transcriptional, post-transcriptional, and translational levels, the vast majority of the mtDNA and RNA-binding proteins involved have been identified. A majority of these proteins belong to a family of RNA-binding proteins characterized by the presence of an N-terminal RRM sequence. They diverge in their C-terminal sequences, suggesting that they can be involved in different plant mt regulation processes (Vermel et al. 2002). Most studies have focused on the developmental roles of some RNA-binding proteins, which encoded by some genes. Here we analyzed the function of two RRMs in *FCA* of rice (r*FCA*).

The developmental transition from vegetative to floral in the life cycle of plant is regulated by multiple environmental and genetic pathways cues. To understand the molecular mechanisms in floral pathways, *FCA* as one component of the autonomous floral pathway have been analyzed in *Arabidopsis*. *FCA* provided an elegant example of post-transcriptional regulation in gene expression and in plant development. The *Arabidopsis* gene *FCA* encodes an RNA binding protein that function to promote the floral translation (Macknight et al. 1997, 2002). *FCA* expression is regulated through alternative processing of its pre-mRNA. *FCA* pre-mRNA is alternatively processed at two positions, resulting in four transcripts: α, β, γ, δ. Transcripts γ encodes the full-length nuclear protein with a WW domain and two RRM-type RNA binding domains that can binding in vitro, (Macknight et al. 1997; Quesada et al. 2003). *FCA* RRM domains are known to mediate RNA-binding functions in a large number of different proteins. The rice *FCA* gene was homologous to FCA-gamma of Arabidopsis and contained conserved domains (two RNA-binding domains and a WW-domain) (Du et al. 2006). We want to provide phenotypic evidence that the two consensus RNA-binding motifs of r*FCA* are functional in rice. The results suggested that the overexpression of RRM1 and RRM2 in transgenic rice have similar functions to regulate gene expression.

Materials and Methods

Construction of Transgenic Expression Vectors

The rice *FCA* RRM1 and RRM2 ORFs (GenBank accession no. AY274928) were cloned in separate plant transformation vectors. In order to prove that RRM play ubiquitous role as regulator for gene expression in rice, two transgenic lines were constructed in the study. The binary plasmid vector pCAMBIA1304 and plasmid

pBY520 were used. Plasmid pCAMBIA1304 contains kanamycin resistance gene as the bacterial selection and the hygromycin phosphotransferase gene for rice transformation selectable maker. pBY520 also has the bacterial phosphinothricin acetyl transferase structural gene (*bar*), which play as a selectable marker (Xu et al. 1996).

Fragment RRM1which subcloned with the *Bgl*II and *Plm*I restriction sites at 5′ and 3′ ends was cloned into the binary plasmid vector pCAMBIA1304 to get recombinant plasmid pCAM-RRM1 (Fig. 1a). In order to obtain the pBY-RRM2 (Fig. 1b), the plasmid pBY520 was digested with *Hin*dIII and *Xba*I and the Fragment RRM2 was inserted between the *Hin*dIII and *Xba*I restricted sites of pBY520.

The recombinant plasmids were transferred into *Escherichia coli* stain DH5α. The pCAM-RRM1 transformants were selected on the LB medium which contained kanamycin (50 mg/l) and the pBY-RRM2 transformants were selected by Carbenicillin (50 mg/l).

Plant Material and Transformation

The rice variety used in this study were *Oryza sativa* L. *subsp. Indica* var. 9311 and *Oryza sativa* L. *subsp. Japonica* var. zhonghua11. Immature seeds of *Oryza sativa* L. *subsp. Indica* var. 9311 were sterilized with 0.1% $HgCl_2$ for 8 min, washed extensively with sterile water, and then immature embryos were cultured on fresh induced medium at 28°C in the dark for callus induction. Rice mature seeds of *Oryza sativa* L. *subsp.*

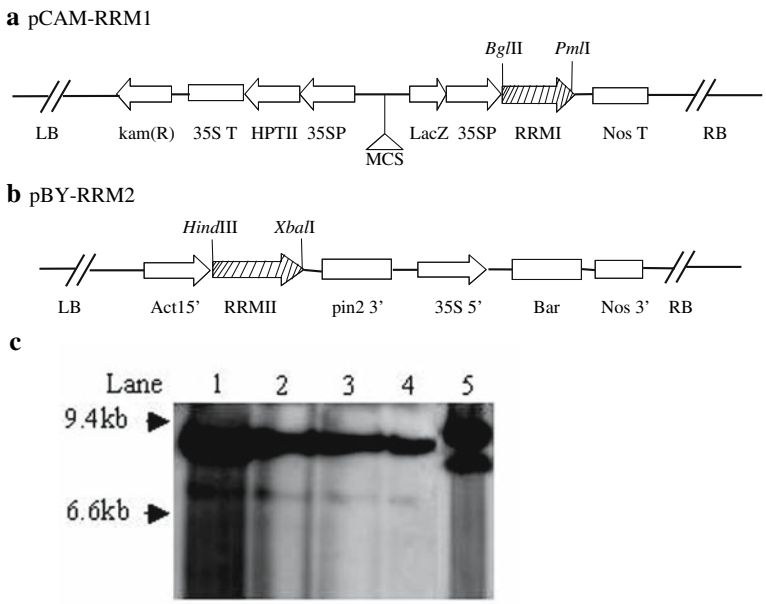

Fig. 1 Transformation of rice by using r*FCA*-RRM1 and r*FCA*-RRM2. (**a**, **b**) Schematic representation of various RRM constructs used to overexpress r*FCA*-RRM1 (pCAMBIA1304:RRM1) and r*FCA*-RRM2 (pBY520:RRM2) in rice. (**c**) Southern hybridization analysis of pCAM-RRM1 and pBY-RRM2 transformants. Lanes 1–4 contain genomic DNA from individual pCAM-RRM1 transformants carrying r*FCA*-RRM1. Lane 5 contains genomic DNA from pBY-RRM2 positive transformants carrying r*FCA*-RRM2. The genomic DNA were digested overnight at 37°C with *Eco*RI and *Hin*dIII. ORFs of r*FCA*-RRM1and r*FCA*-RRM2 were used to be probe, respectively

Japonica var. zhonghua11 were dehusked and surface sterilized by immersion in 70% ethanol for 3 min and then shaking in a 0.1% solution of $HgCl_2$ for 20 min and washing in sterilized water for four times. The seeds were placed on sterilized filter papers to dry and then inoculated on medium for embryogenic callus (Attia et al. 2005). Embryogenic calli were selected after 15 days subculture and used as the transformation material. Callus which been derived from immature embryos of *Oryza sativa* L. *subsp. Indica* var. 9311 were bombarded with tungsten particles coated with the pCAM-RRM1 plasmid. Resistant callus were selected in selection medium, containing 5 mg/l hygromycin as the selective agent, for 2–3 weeks. Suspension resistant callus were transferred into regeneration medium to grow into plants in the greenhouse. pBY-RRM2 were introduced into the embryogenic callus of *Oryza sativa* L. *subsp. Japonica* var. zhonghua11.by microprojectile bombardment and selected with 5 mg/l phosphinothricin.

PCR Analysis to Identify the RRM1 and RRM2

Genomic DNA was extracted from leaf tissue of wild type and transgenic rice as template as described (Sheu et al. 1996). In transgenic line pCAM-RRM1, the primers 5′-ATGGTAGATCTATGGGCGGC-3′ and 5′-ATTCACACGTGTCACTGGTTG-3′ were used for amplification. pBY-RRM2 was checked by primers 5′-AC-CAAGCTTATTTAGGTGACACTATAGAA-3′ and 5′-TCCTCTAGATAATACCG-ACTCACTATAG-3′. The primers contained the restriction sites and the sequences of plasmid vector pCAMBIA1304, pBY520 respectively.

Southern Blot Analysis

For Southern hybridization, 20 μg of total genomic DNA from leaf tissue of transgenic and control plants were digested with appropriate restriction endonucleases. The DNA fragments were separated by electrophoresis in 0.8% agarose gels and then transferred to nylon membranes (Amersham Bioscience) according to the standard protocol. The probes for Southern hybridization were fragments respectively of the RRM1 and RRM2 coding sequences.

Analysis of Morphologies in Transgenic Rice

The seed length and width of control plant and transgenic plant were measured. The Mature pollen collected from wide-type and transgenic plants were stained by kalium iodide solution and then viewed on the light microscope and photographed.

Analysis of Plants Cell Size

The sheath, leaf tissues of plants from wide-type, transgenic lines of pCAM-RRM1 and pBY-RRM2 were used for histological analysis. Whole grains and leaf tissues of rice were dehydrated in a graded ethanol series (30, 40, 50, 60, 70, 80, 90, 95% × 2, 100% × 2) followed by xylene, 1 day per step, at 21°C. The grains and leaf tissues were transferred to melted paraffin at 70°C and allowed to infiltrate for 1 day. The resulting paraffinized grains and leaf tissues were placed in embedding molds with additional paraffin and chilled to harden the paraffin. Before the paraffin was completely hardened, the microtome chuck was gently placed on top of the mold to secure the block for sectioning.

Embedded rice sheath, leaf tissues were sectioned at ambient temperature using a rotary microtome (CM 3000 cryostat, Leica, Germany) equipped with disposable (Leica Model 819) blades. While holding the uppermost edge of the tape, the microtome was advanced to cut a 10 μm section, which adhered to the tape. The taped section was affixed to a glass slide with the specimen side facing up using a thin strip of adhesive tape. The sections were deparaffinized in xylene for 1 h. After that, these sections were stained and then faded, dried and viewed on the light microscope and photographed (Tomasi and Rovasio 1997; Ogawa et al. 2003).

Results

RNA recognition motif proteins have roles in RNA processing and transport, regulation of RNA stability, and translational control in most organisms. Various molecular mechanisms of RNA-binding activities in plant cells are very complex. In this context, we have previously shown that overexpression of RRM1 and RRM2 of rice *FCA* in two variety rice results in similar morphology modification in transgenic plants. This finding indicates that the introduce trait of rice *FCA* RRM1 and RRM2 is functionally and genetically stable.

Expression of RRM1 and RRM2 in Rice (*Oryza sativa* L. *subsp. Indica* var. 9311 and *Oryza sativa* L. *subsp. Japonica* var. zhonghua11)

Plasmid pCAM-RRM1 and pBY-RRM2 containing r*FCA* RRM1 and r*FCA* RRM2 were introduced into the rice *Oryza sativa* L. *subsp. Indica* var. 9311and *Oryza sativa* L. *subsp. Japonica* var. zhonghua11, respectively. Transgenic lines of pCAM-RRM1 and pBY-RRM2 transformants, growing on the antibiotic selection medium, were screened by using hygromycin and phosphinothricin, respectively. The transgenic nature of the plants was also checked by PCR using rice genomic DNA as template and specific primers (as date shown). PCR-positive plants were further confirmed for the stable integration of the transgenes by Southern hybridization using equal amounts of transgenic rice genomic DNA digested with *Eco*RI and *Hin*dIII and probed with rice *FCA* RRM1 and RRM2 ORFs. The specific signal band in two transgenic lines of pCAM-RRM1 and pBY-RRM2 plants was observed (Fig. 1c).

Constitute Expression of RRM1 and RRM2 Affect Similar Morphology Alterations in Two Transgenic Lines Plants

We examined whether RRM proteins, r*FCA*-RRM1 and r*FCA*-RRM2 caused any similar effect on the development of the transgenic plants by regulating the gene expression. In transgenic lines, pCAM-RRM1 and pBY-RRM2, the vegetative stage and growth of transgenic rice plants was retarded compared to the wide-type rice. Transgenic plants were relatively dark green, wider and thicker leaves. It was indicated that the two transgenic lines plants had larger seeds and larger pollen (Figs. 2, 3). Transgenic T_1 plants derived from self-pollinated T_0 plants had same phenotypes, heading time and normal fertility as the control plants. It was suggested that the phenotypic variance of transgenic T_0 plants was transmitted genetically to the next generation in the two expression systems. These results indicated that the same phenotypic characteristics of late flowering and increased grain volume in the two

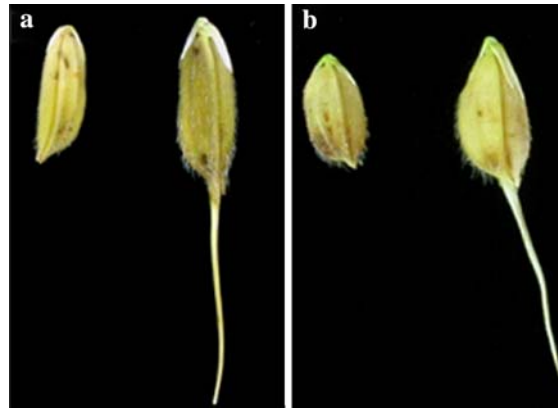

Fig. 2 pCAM-RRM1 and pBY-RRM2 transgenic lines produce large seeds. Shown are mature dried seeds from *Oryza sativa* L. *subsp. Indica* var. 9311wild type, pCAM-RRM1developing seed (**a**) and *Oryza sativa* L. *subsp. Japonica* var. zhonghua11 wild type, pBY-RRM2 developing seed (**b**)

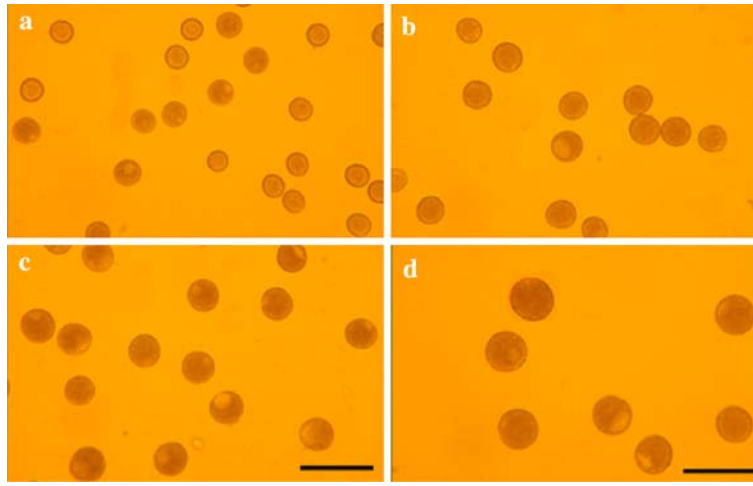

Fig. 3 r*FCA*-RRMs regulate rice pollen size. *Oryza sativa* L. *subsp. Indica* var. 9311wild type and *Oryza sativa* L. *subsp. Japonica* var. zhonghua11 wild type (**a**, **b**), pCAM-RRM1developing pollen and pBY-RRM2 developing pollen (**c**, **d**) were stained by kalium iodide and then viewed on the light microscope and photographed. And the morphology modification in pollen is the similar results to seeds development. Bar = 100 μm

transgenic lines associated respectively with the expression of the r*FCA* RRM1 and r*FCA* RRM2 transgene.

Expression of RRM1 and RRM2 Gives Rise to Seed Size

Seed development involves complex processes, including the expansion and growth of the maternal integuments of the ovule, integrated growth and development of the genetically diverse integument embryo, and endosperm tissues. The endosperm plays a key role in controlling seed size in both monocots and dicots (Lou et al. 2005). For the

larger size of seed, we discovered that sheath, endosperm cells size of r*FCA*-RRM1 and r*FCA*-RRM2 were equivalent larger than wide-type plant. These most similar morphology shown that the two RNA binding domains, r*FCA*-RRM1 and r*FCA*-RRM2 play the similar role in the rice seed development.

We measured the length and width of dried mature seeds from transgenic T_0, T_1 plants and wide-type plants. It was shown that there were 26, 28.6% increase in average length and 16.6, 17% increase average width of pCAM-RRM1 and pBY-RRM2 transgenic lines compared with wide-type plants (Table 1). The weights of seeds from T_0 pCAM-RRM1 and pBY-RRM2 transgenic lines were measured. We found that pCAM-RRM1 and pBY-RRM2 produced seeds were heavier than those from wide-type plants. Specially, pCAM-RRM1 and pBY-RRM2 seeds were1.5, 1.73 times heavier, respectively, than wide-type seeds (Table 1). Similar results were obtained for seeds formed on T_1, T_2 generations. Therefore, the effect of each RRM expression system on seed weight corrected with the overexpression induced morphologies modifications on rice. That is, pCAM-RRM1 and pBY-RRM2 produced larger seeds, suggesting that activity of r*FCA* RRM1 and r*FCA* RRM2 is responsible for the development of rice seed mass.

Expression of RRM1 and RRM2 Controls Cell Size

To analyze the phenotype of the transgenic rice plants at the cellular level, the histological analysis of sheath cells from pCAM-RRM1, leaf tissues of pBY-RRM2 and wide-type, plants were done, respectively. We observed that pCAM-RRM1 sheath cells were significantly larger than those of wild type (Fig. 4). In addition, imaging experiments showed that the average area of pCAM-RRM1 sheath cells was 1.5 times larger than that of wild type. We also found that cells from pBY-RRM2 leaf tissues were larger than those of wide type (Fig. 4). The average area of a pBY-RRM2 leaf cell was 1.73 times larger than that of a corresponding wild-type cell. Our data showed that the sheath and leaf cells size of the transgenic plants were fully larger than those of wide-type plants. Given differences in the areas of pCAM-RRM1and pBY-RRM2 transgenic plants, we conclude that overexpression of RNA binding domains must affect cell size, with transgenic plants having $\approx$1.6 times more cells than wild type. These results suggested that overexpression of the r*FCA* RRM1 and r*FCA* RRM2 caused cell size rise of transgenic rice in vegetative and reproductive stages.

Discussion

FCA is a nuclear RNA-binding protein that promotes flowing by preventing the accumulation of mRNA encoding FCL, a MADS box transcription factor that is a

Table 1 pCAM-RRM1 and pBY-RRM2 transgenic plants produce large seeds

Genotype	Seed length (mm)	Seed width (mm)	Seed weight (g)
Wide-type 9311	8.20 ± 0.12	2.71 ± 0.14	2.78 ± 0.1
Wide-type zhonghua11	7.34 ± 0.14	3.23 ± 0.09	2.23 ± 0.1
pCAM1304:RRM1	10.36 ± 0.14	3.16 ± 0.12	4.13 ± 0.12
pBY520:RRM2	9.44 ± 0.23	3.78 ± 0.13	3.81 ± 0.23

Seeds weight is given for 100 dry grains of T_0 lines from pCAM1304:RRM1, pBY520:RRM2 transgenic plants lines

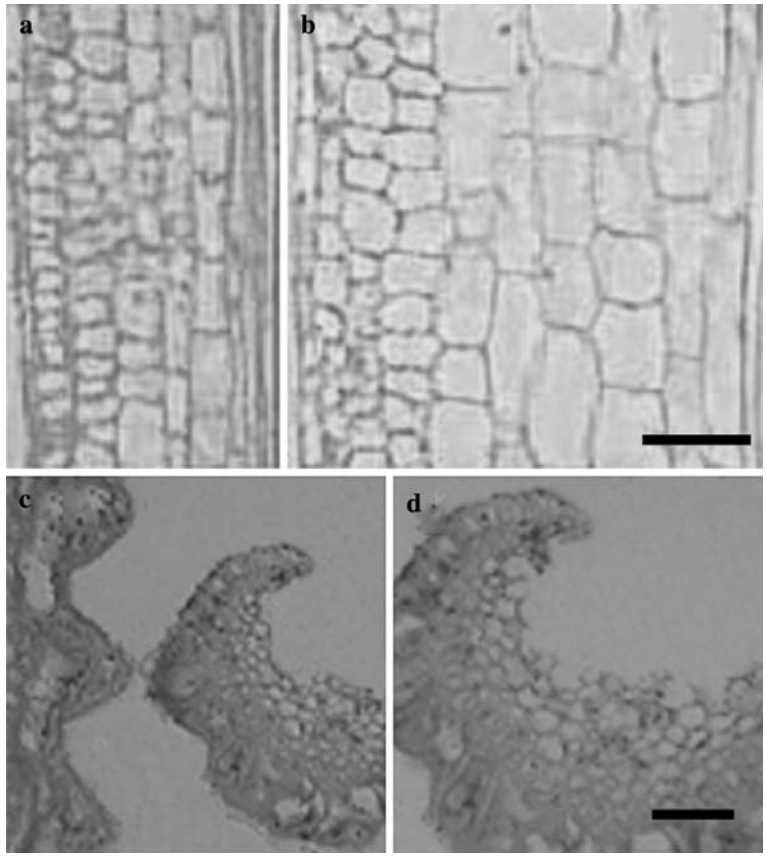

Fig. 4 r*FCA*-RRMs overexpression causes an increase in both sheath cell size and leaf cell size. (**a, c**) Young sheath and leaf from wide type and pCAM-RRM1 and pBY-RRM2 transgenic lines (**b**, **d**) were dissected, cleared, fixated, dehydrated, and embed for histological analysis. Bar = 200 μm

potent repressor of the floral transition (Henderson and Dean 2004; Simpson 2004; Michaels and Amasion 1999; Sheldon et al. 2000). *FCA* autoregulates its expression by promoting premature cleavage and polyadenylation in intron 3 of its own precursor mRNA (Macknight et al. 2002; Quesada et al. 2003). This function of *FCA* also requires interaction with the RNA 3′-end processing factor FY. FY binds to its tryptophan–tryptophan (WW) protein interaction domain (Simpson et al. 2003). The *Arabidopsis* genome encodes many RNA-binding proteins, including 198 that possess the most common RNA-binding protein domains and half of these RRM-containing proteins are uncharacterized and plant-specific (Cheng and Chen 2004). Several plant RNA-binding proteins have been studied. The function of RRM proteins can be predicted based on the similarity with their metazoan counterparts. The overexpression of AtSRp30 in Arabidopsis leads to alternative RNA processing and delays developmental transitions (Lopato et al. 1999). The two RNA binding motifs in *FCA* similarity to RNA binding region in Sxl. Sxl regulates alternative splicing of other genes in the sex determination pathway (Smith et al. 1989). *AP2* in *Arabidopsis* is a transcription factor affect seed mass by regulating the expression of other genes (Ohto et al. 2005). These findings indicate the existence of RRM proteins machineries roles.

RNA-binding protein as a regulator of plant development is that the protein regulates the expression of its target genes, resulting in temporally or spatially distinct patterns of genes expression during development or in response to environmental stimulators (Cheng and Chen 2004). Recently, the abundance of works on *Arabidopsis* genes encoding RNA-binding proteins, and the identification of developmental and hormone-response mutations in *Arabidopsis* genes that encode RNA-binding proteins, suggests that RNA-binding proteins are important payers in plant morphogenesis and cellular regulation (Fedoroff 2002). The *FCA* gene plays an important role in floral development of higher plants. The *FCA* gene function and mechanism of action regarding the role(s) and components of the autonomous pathway and the role of alternative splicing in the regulation of gene expression, meristem function, and the transition to flowering. In our study, r*FCA* RRM1 and r*FCA* RRM2 as transcription factors, may involved in regulation of the gene expression by affecting the flowering time and cell size in rice. The two proteins may play a permissive role in the expression of their target genes, or as key developmental regulators in gene expression pathway. The regulation of gene expression at post-transcriptional levels cause the production of functional proteins, lead to the generation of protein isoforms to gene developmental hints. It was identified that Many RNA-binding proteins have potential roles in post-transcriptional regulation of plant development. Our results implicated that overexpression of r*FCA* RRM1 and r*FCA* RRM2 caused plant organs, including seeds, to increase in size in transgenic rice. Thus, r*FCA* RRM1 and r*FCA* RRM2 proteins may function in a similar pathway to regulate gene expression in transgenic plants.

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